

# Integration of the Receptor for Bacteriophage Lambda in the Outer Membrane of *Escherichia coli*: Coupling with Cell Division

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Induction of the synthesis of the receptor for phage  $\lambda$  is obtained by adding maltose and adenosine 3'-5'-cyclic monophosphate to glucose grown cells of *Escherichia coli*. Bacteria induced for a short period of time were infected with a high multiplicity of phage  $\lambda$ , and examined under the electron microscope. Only a fraction of the bacteria were seen to have adsorbed a large number of phage particles. The majority of such bacteria had a constriction indicating formation of a septum, and, in this case, the density of adsorbed particles was highest in the vicinity of the constriction. When found on bacteria showing no sign of septum formation, the adsorbed particles were asymmetrically distributed, one pole of the bacteria being more heavily covered with phage particles than the other. Such asymmetrically covered bacteria are believed to have originated from cells which divided during the induction period. The results suggest that the receptor for phage  $\lambda$ , a protein of the outer membrane, is integrated in the cell envelope during the last quarter of each generation and that the integration process is initiated in the vicinity of the forming septum.

Little is known about the biosynthesis of the outer membrane of gram-negative bacteria (3). As an approach to this problem we decided to follow the appearance at the cell surface of a particular protein of the outer membrane of *Escherichia coli*, the receptor for bacteriophage  $\lambda$ . The  $\lambda$  receptor protein (15) is the product of gene *lamB* (18, 20; unpublished data) which happens to be located in one of the maltose operons (5). Its synthesis is induced by maltose (18) and is subject to catabolite repression (13, 21). Simultaneous addition of maltose and adenosine 3'-5'-cyclic monophosphate to glucose-grown cells thus very efficiently induces  $\lambda$  receptor synthesis. Appearance at the cell surface of  $\lambda$  receptor molecules synthesized during short periods of induction can be followed by adsorbing phage  $\lambda$  particles to the bacteria and by examining the infected bacteria under the electron microscope. The present work demonstrates that  $\lambda$  receptor molecules synthesized during short periods of induction are not randomly distributed, either among the different bacteria of the culture or on the surface of individual bacteria.

## MATERIALS AND METHODS

**Assay of  $\lambda$  receptor.** A stock of  $\lambda$ Vh is diluted in a  $10^{-2}$  M  $\text{MgSO}_4$  solution to a final titer of  $3 \times 10^8$  to  $6$

$\times 10^8$  plaque-forming units (PFU) per ml. The receptor containing extract is diluted in  $10^{-2}$  M Tris buffer, pH 7.5. When equal volumes of receptor solution and phage suspension are incubated at 37 C, the phage titer decreases according to first-order kinetics. The first-order rate constant is proportional to receptor concentration (15).

**Preparation of infected cells for electron microscopy.** Cells of *Escherichia coli* K-12, strain 3000, were inoculated in medium M63 (12) supplemented with thiamine (0.0005%) and glucose ( $2 \times 10^{-2}$  M), and grown at 37 C for at least 15 generations. Before starting the experiment, the cells were reinoculated in the same medium and grown for 3 to 4 h so that they underwent three to four doublings and reached a density of  $2 \times 10^8$  bacteria/ml. Maltose ( $10^{-2}$  M) and adenosine 3'-5'-cyclic monophosphate ( $4 \times 10^{-3}$  M) were then added. These additions had a slight inhibitory effect on bacterial growth, the generation time changing from 60 to 70 min (see Fig. 1a). Samples (1 ml) were withdrawn at different times, and transferred into tubes (kept in ice) containing 50  $\mu$ l of a solution of chloramphenicol (2.5 mg/ml). (Chloramphenicol was used in the experiment reported here, but essentially identical results were obtained when the drug was omitted). The samples were then centrifuged, and the cells were suspended in 100  $\mu$ l of cold  $10^{-2}$  M  $\text{MgSO}_4$  solution supplemented with 50  $\mu$ g of chloramphenicol per ml, and mixed with 25  $\mu$ l of a  $\lambda$ Vh (15) stock which had been purified on CsCl gradients and which titered  $1.5 \times 10^{12}$  PFU/ml. The multiplicity of infection was thus about 200 active

particles per bacterium. The samples were then incubated for 7 min at 37 C to allow adsorption of the phage, and centrifuged for 1.5 min in a 152 Beckman Microfuge to remove most of the unadsorbed phage. The cells were suspended in 50  $\mu$ l of  $10^{-2}$  M  $\text{MgSO}_4$  solution, and 50  $\mu$ l of 1% formaldehyde was immediately added. Five minutes later the bacteria were deposited on carbonized formvar-coated grids which had previously been submitted to a glow discharge. The preparations were stained with 1% uranyl acetate, and examined under a Siemens Elmiskop 101 electron microscope.

In the text, for the sake of simplicity, the number of phage particles adsorbed on a bacterium is equated to the number of receptor sites carried by this bacterium. This may not be the case, because, for kinetic or stoichiometric reasons, all receptor sites may not have reacted with phage. However, since only relative numbers are considered in the experiments, this simplification should be admissible.

## RESULTS

Lambda receptor synthesis can be induced in *E. coli* K-12 by adding maltose and adenosine 3'-5'-cyclic monophosphate to glucose-grown cultures (Fig. 1). An absolute lag of about 4 to 5 min is observed, and the amount of receptor then increases linearly with cell mass.

Cultures which had been induced for various periods of time were infected with a high multiplicity of lambda phage (about 200 active particles per bacterium) and examined under the electron microscope. In cultures induced for more than one generation all bacteria are covered with a large number of phages particles homogeneously distributed at the surface of each individual bacterium (Fig. 2a). Very different situations are observed in uninduced cultures, and in cultures induced for a short period of time.

Figure 2b shows a typical picture obtained from an uninduced culture. The number of adsorbed phage particles varies very strikingly from one bacterium to the other. The heterogeneity of an uninduced culture (already reported by Howes [6]) is further illustrated in Fig. 3, which gives the distribution of bacteria according to the number of phage particles they have adsorbed. The histogram is clearly not unimodal and shows that about 7% of the bacteria carry 70 to 90 phage particles, while most of them carry fewer than 30. This heterogeneous distribution suggests that integration of the receptor in the cell envelope is a discontinuous process, occurring about every third generation or even less frequently. Most of the time the phage particles are homogeneously distributed at the surface of individual bacteria.

After 9 min of induction with adenosine

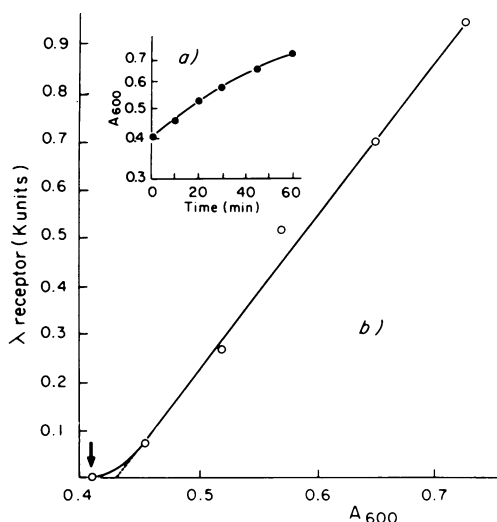


FIG. 1. Induction of  $\lambda$  receptor synthesis as measured in cell extracts. Induction was realized as described in Materials and Methods. The arrow indicates the time of maltose and adenosine 3'-5'-cyclic monophosphate addition. At the times indicated, 1-ml samples were taken into 50  $\mu$ g of chloramphenicol per ml at 4 C, their absorbancy at 600 nm ( $A_{600}$ ) was measured, and they were centrifuged. The pellets were suspended in 100  $\mu$ l of  $10^{-2}$  M tris(hydroxymethyl)aminomethane  $2 \times 10^{-3}$  M ethylenediaminetetraacetate, 2% sodium cholate and incubated for 30 min at 37 C, for extraction of the  $\lambda$  receptor. (a) The  $A_{600}$  of the samples is plotted versus the time of sampling. (b) The  $\lambda$  receptor activity (15) in the extracts is plotted versus the  $A_{600}$  of the corresponding samples.

3'-5'-cyclic monophosphate and maltose, two additional classes of bacteria are found: bacteria starting to divide on which adsorbed phage particles are concentrated around the septum (Fig. 4a and b) and bacteria showing no sign of division displaying a high concentration of particles at one pole (Fig. 4d). The heterogeneity of the distribution of phages particles at the surface of these classes of bacteria is further illustrated in Fig. 5 where the results from examination of several bacteria are pooled. It is tempting to argue that the bacteria from the second class (one pole labeled) originated from bacteria of the first class (septum labeled). The data in Fig. 6 favor this hypothesis. First, the frequency of bacteria labeled at one pole is higher (28%) among small, presumably newborn, cells (2-3  $\mu$ m) than among large cells (3-4  $\mu$ m) showing no sign of division (18%). The fact that polarly labeled bacteria are not exclusively found in small cells is not too surprising since the size of cells at division was far from homoge-

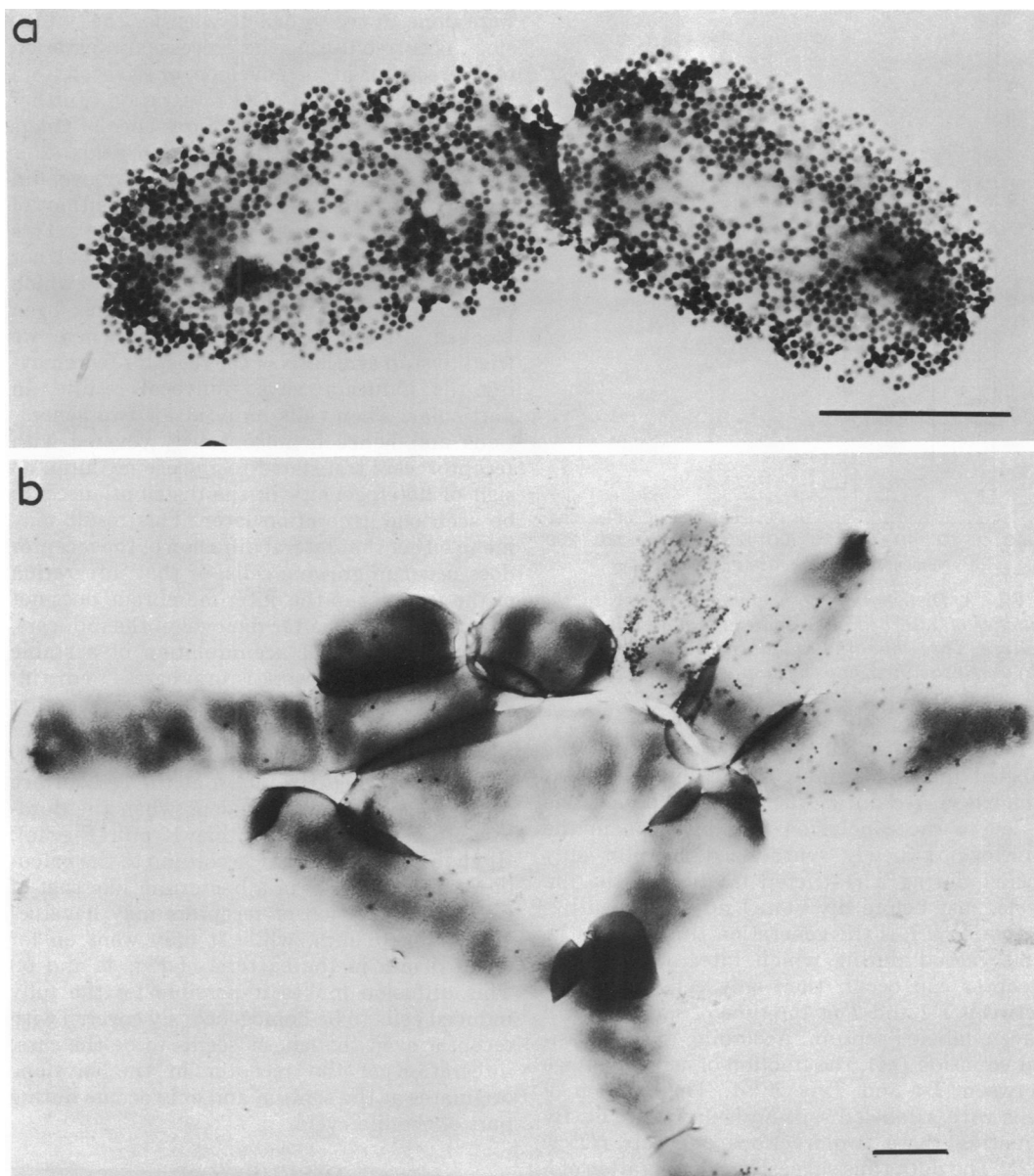


FIG. 2. Distribution of  $\lambda$  receptor at the surface of fully induced and uninduced bacteria. (a) Fully induced bacteria. The bacteria were grown for several generations in M63 maltose, harvested in exponential phase, centrifuged and infected with a high multiplicity of phage, and examined under the electron microscope as described in Materials and Methods. (b) Uninduced bacteria. Same as (a) except that the bacteria were grown in M63 glucose. The bars represent 1  $\mu$ m.

neous in our culture. Second, the total frequency (12%) of cells labeled at one pole is close to the frequency of bacteria expected to have originated in a division event occurring during the period of receptor synthesis. This period must be taken as being about 5 min, since (Fig.

1; checked by electron microscopy) there is a 4- to 5-min lag before induced receptor can be detected in the culture. In a culture of bacteria growing exponentially with a generation time of 60 min, 11.2% of the bacteria are between 0 and 5 min old (14).

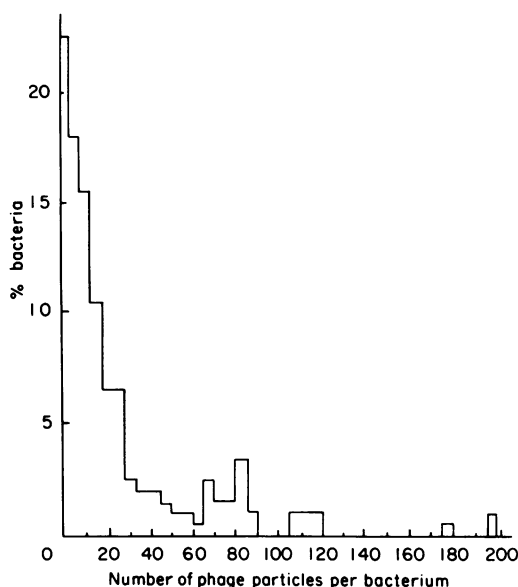


FIG. 3. Distribution of bacteria according to the number of adsorbed phage particles in an uninduced culture. Three hundred uninduced bacteria (see Fig. 2b) were examined, and the number of phage particles adsorbed on each of them was counted.

If one assumes that the bacteria with one pole labeled indeed originated in a division event which occurred during the induction period, one is led to the conclusion that insertion in the envelope of newly synthesized receptor only occurs during a restricted fraction of the life cycle, just before division. Under such an hypothesis, if  $T$  is the generation time, and  $t$  the time period during which integration of the receptor can occur, then only cells of an age between  $T-t$  and  $T$  at the time of sampling will have a labeled septum. According to the classical equation (14), the fraction of cells of an age between  $T-t$  and  $T$  is  $2^{t/T}-1$ . The fraction of cells with a labeled septum is 0.17 (Fig. 6). By equating those two fractions one gets  $t/T = 0.23$ . The data thus suggest that integration of  $\lambda$  receptor in the outer membrane only occurs during the last quarter of each generation.

If one assumes that the lateral surface of the bacteria increases in a continuous manner, like the bacterial mass, the relative increase in lateral surface during the 5-min period of receptor synthesis is at most 10%. However, the area in which newly synthesized receptor was integrated is much larger (Fig. 4 and 5), certainly more than 30% of the bacterial surface. This result suggests that lambda receptor originates from the septum region and then in some way "diffuses" towards the poles. Some experiments

were done to try to decide whether the "diffusion" occurred during the process of integration of the receptor in the envelope, or resulted from true lateral diffusion after integration. Further incubation for 15 min in the presence of chloramphenicol (100  $\mu\text{g/ml}$ ) or sodium azide ( $2 \times 10^{-2}$  M) of cells induced for 9 min as above, did not significantly change the distribution of phage at the bacterial surface (Fig. 4e). This result suggests that lambda receptor does not diffuse freely at the surface of bacteria in which protein synthesis or energy supply has been blocked. Preliminary experiments, where we tried to stop synthesis of the receptor by removing the inducers, gave equivocal results. In particular, when cells induced for two generations and hence homogeneously covered with receptor were transferred to glucose medium, no sign of heterogeneity in the distribution could be seen one generation later. This result may mean either that lateral diffusion of the receptor does occur in growing cells, or that integration of the receptor in the outer membrane does not stop immediately after removal of the inducers, perhaps because of accumulation of a stable precursor. Further work is in progress to distinguish between these two possibilities. Whether it occurs during or after integration in the outer membrane, the diffusion process is very efficient, since the distribution at the cell surface rapidly becomes homogeneous when the duration of induction is longer than 10 min (Fig. 4c). (It should be noted that according to the calculation made above, in a bacterium like that of Fig. 4c, integration of receptors may have occurred for 15 min, while it only went on for about 5 min in the bacteria of Fig. 4a and b.) This diffusion makes it possible for the fully induced cells to be homogeneously covered with receptor even though, as seems to be the case, integration of the receptor in the envelope originates at the septum and only occurs during part of the life cycle.

## DISCUSSION

After  $\lambda$  receptor synthesis has been induced for a short period of time, an increase in the amount of receptor sites is seen on the surface of only a fraction of the bacterial population. This fraction comprises bacteria of two types. Bacteria of the first type have a visible constriction indicating a forming septum, and display a high concentration of receptors in the vicinity of this constriction. Bacteria of the second type show no sign of division and have a high concentration of receptors at one pole. For reasons discussed above, we believe that bacteria from this

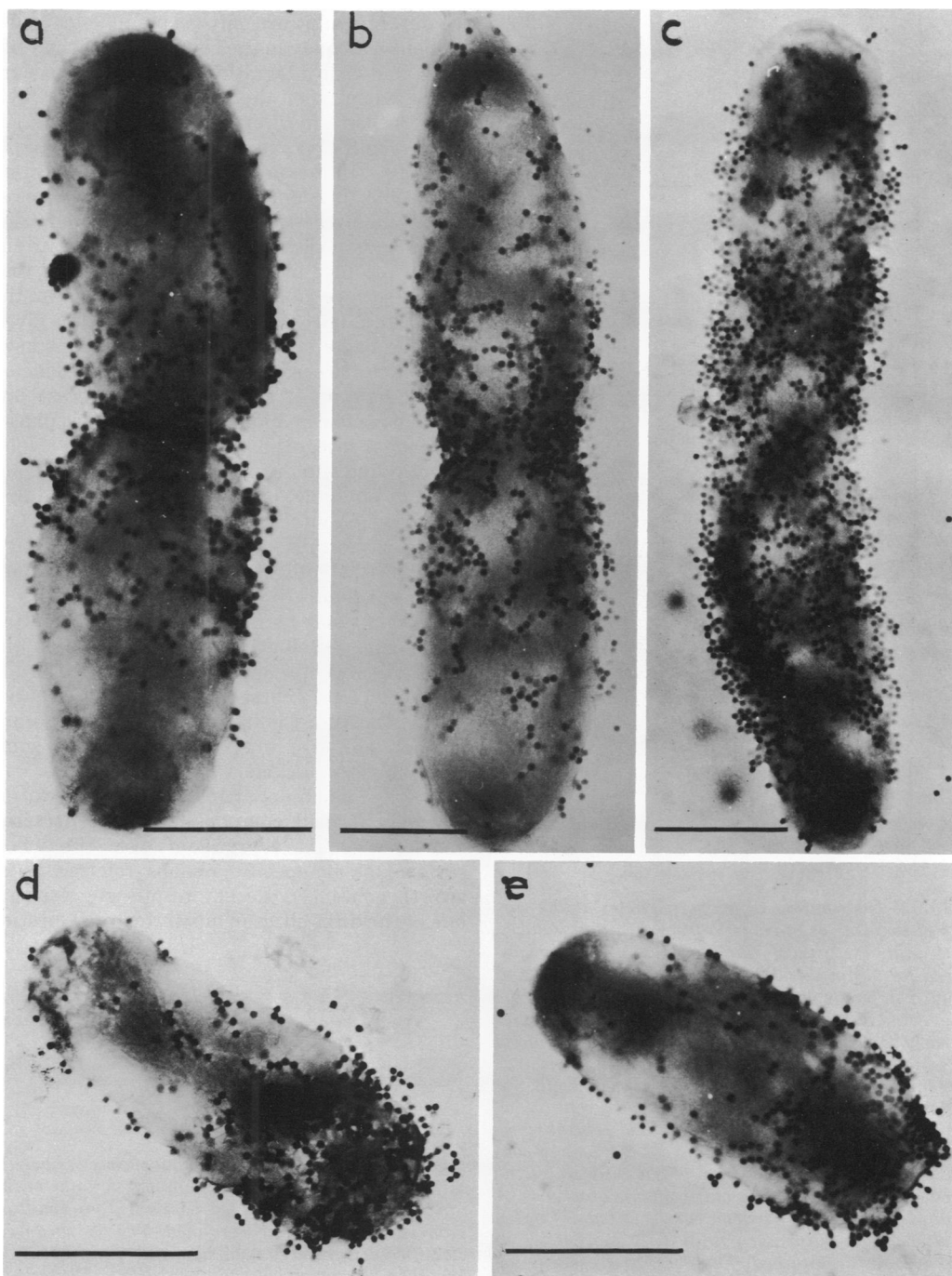


FIG. 4. Distribution of  $\lambda$  receptor at the surface of bacteria induced for a short period of time. The bacteria were grown and infected as described in Materials and Methods. (a,b,d) Bacteria from a culture induced for 9 min; (e) Bacterium from a culture induced for 9 min and further incubated for 15 min in the presence of  $2 \times 10^{-2}$  M sodium azide; (c) Bacterium from a culture induced for 20 min. The bars represent 1  $\mu\text{m}$ .

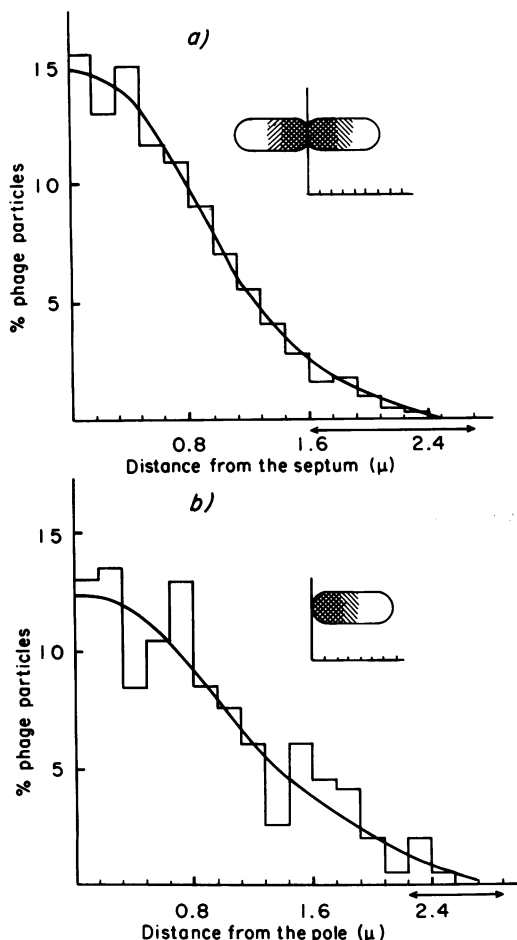


FIG. 5. Distribution of phage particles at the surface of two classes of bacteria from a culture induced for 9 min. Pictures of bacteria from the culture induced for 9 min (see Fig. 4 a,b,d) were enlarged and divided in bands of equal width, perpendicular to the axis of the bacterium. The number of phage particles in each band was counted. The results from several bacteria of the same class were pooled and plotted as percentage of total phage found in each band. (a) Eighteen dividing bacteria with a labeled septum (as shown in Fig. 4 a and b) were examined. The abscissa gives the distance of each band from the zone of constriction. The arrow under the abscissa indicates the fluctuation in size of the half-bacteria observed. A total of 3,300 phage particles was counted. (b) Seven small bacteria with one pole heavily labeled (as in Fig. 4 d) were examined. The abscissa gives the distance of each band from the most heavily labeled pole. The arrow indicates the size fluctuation of the bacteria examined. A total number of 400 phage particles was counted.

second class originated from bacteria which divided during the induction period. We therefore propose that integration of the receptor in

the outer membranes only occurs in "old" cells, i.e. cells which are in the last quarter of their life cycle, and that integration preferentially occurs in the vicinity of the forming septum. Obviously, other interpretations could be offered, which would also explain some of the results. It could be, for instance, that old cells are more permeable to adenosine 3'-5'-cyclic monophosphate and are hence more readily induced. This would explain the heterogeneity in the distribution of receptor in the population, but not that at the surface of individual bacteria. On the other hand, only presumptive evidence is given that the bacteria labeled at one pole are newborn bacteria originating from parents labeled at the septum. If this were not the case one would have to conclude that there are two phases of receptor integration, one when the cell is relatively young and during which integration takes place at one pole, one when the cell is old and during which integration takes place in the vicinity of the forming septum. Such a model would resemble that proposed by Donachie and Begg (4) for envelope growth.

There are some other reports in the literature that some proteins may be integrated in the bacterial envelope only at the end of the life cycle (11,17,19). These results imply that either the synthesis or the insertion in the membrane of such proteins must be regulated by some event in the cell cycle.

There is much debate on whether integration of newly formed components of the bacterial envelope occurs at random over the whole surface, or in specific regions referred to as growth zones (7). Recent results suggest that one of the difficulties in answering this question

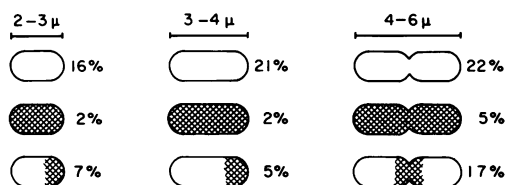


FIG. 6. Distribution of the various classes of bacteria after 9 min of  $\lambda$  receptor induction. A total of 360 randomly chosen,  $\lambda$ -infected bacteria from a culture induced for 9 min were examined. The bacteria were classified according to their size, and to the density of adsorbed phage particles on their surface. Bacteria on the top line carry less than 50 phage particles, homogeneously distributed. Bacteria on the middle line carry more than 50 phage particles, homogeneously distributed. These two types of bacteria are also found in uninduced cultures (Fig. 2b). Bacteria on the bottom line have a heterogeneous distribution of particles at their surface, as illustrated in Fig. 4 a, b, and d.

stems from the fact that the situation may not be the same for the different components of the envelope. For instance, while new peptidoglycan seems to appear preferentially in the central part of the bacterium (16), the lipopolysaccharide would be deposited over the whole surface (9,10), around the membrane-cell wall adhesion sites described by Bayer (1). The results presented here are ambivalent. Insertion of the receptor in the envelope appears to be nonrandom after short periods of induction, and random after long periods. Considering our evidence that there is no redistribution of the receptor over the surface once it is inserted, the following model can be proposed. The receptor would be exclusively synthesized in the vicinity of the forming septum, and/or would cross the peptidoglycan exclusively in that region. It would then diffuse in the periplasmic space until it finds one of a limited number of sites where it could get inserted in the outer membrane. The closer such a site to the septum, the sooner it is likely to be filled with a receptor molecule. According to such a model the randomness observed after long periods of induction would result from the fact that the bacteria were originally devoid of receptor, and hence had all these sites in a free state. On the other hand cells growing exponentially under conditions of complete induction would be expected to insert the newly synthesized receptor in a much more restricted area in the vicinity of their septum.

Leal and Marcovich (8; Mol. Gen. Genet., in press) came to conclusions similar to ours with respect to the receptor for phage T6. In their case, however, it is not known whether the starting bacteria are altogether devoid of T6 receptor molecules, or have modified, inactive T6 receptor molecules in their envelope. Begg and Donachie (2) suggest that the outer membrane, as defined by its sites for insertion of T6 receptor, grows from the poles rather than the center. Their results are difficult to compare with ours, however, because they are working with bacteria in which division was inhibited by low amounts of penicillin.

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